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Cloning and Characterization of a New Type of Fimbria (S/F1C-Related Fimbria) Expressed by an *Escherichia coli* O75:K1:H7 Blood Culture Isolate

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The *Escherichia coli* blood culture isolate BK658 (O75:K1:H7) expresses F1A and F1B fimbriae as well as a third fimbrial type which reacts with anti-S-fimbrial antiserum but fails to show S-specific binding properties (i.e., agglutination of bovine erythrocytes). To characterize these fimbriae, we cloned the respective genetic determinant in *E. coli* K-12. The resulting recombinant clone HB101(pMMP658-6) expresses fimbriae of 1.2-μm length and a diameter of approximately 7 nm. The determinant codes for the fimbrillin subunit, a protein of 17 kilodaltons in size, and for at least five other proteins of 87, 31, 23, 14.3, and 13.8 kilodaltons. By restriction analysis and by DNA-DNA hybridization, it could be shown that the cloned fimbrial determinant of strain BK658 exhibits a high degree of sequence homology to the gene clusters coding for S fimbrial adhesins (*sfa*) and F1C fimbriae (*foc*). By using the Western blot (immunoblot) technique and a quantitative enzyme-linked immunosorbent assay, it could be further demonstrated that the cloned fimbriae of BK658, S fimbriae, and F1C fimbriae share cross-reactive epitopes as well as antigenic determinants specific for each fimbrial type. No antigenic cross-reactivity with F1C fimbriae could be detected. The results indicate a genetical and serological relatedness of the cloned fimbriae to S fimbriae and F1C fimbriae. Therefore, this new type of fimbriae is preliminarily termed S/F1C-related fimbriae (Sfr).

Escherichia coli strains that cause extraintestinal infections (urinary tract infections, UTI, septicemia, and newborn meningitis) are frequently found to be associated with specific virulence factors that enable the bacteria to survive and multiply in the host (32). These virulence factors include capsule production, especially of type K1; aerobactin synthesis; and hemolysin production, as well as the expression of fimbriae (7, 14, 20, 22). Fimbria-associated adhesins are known to mediate the attachment of bacteria to various eucaryotic cells. Depending on their binding specificity for α-D-mannose, they are classified into mannose-sensitive (MS) and mannose-resistant (MR) fimbriae (30, 33). MS fimbriae (also termed type 1 or F1A fimbriae) represent a serologically and genetically homogeneous group of adhesins, and they are found on pathogenic as well as on nonpathogenic *E. coli* isolates (2, 33). In contrast, the presence of MR fimbriae is strongly related to pathogenic strains (6). On the basis of their receptor specificity, MR fimbriae of extraintestinal *E. coli* pathogens are subdivided into different groups. One main group of MR fimbriae, P fimbriae, is known to bind to the α-D-galactose-(1-4)-β-D-galactose digalactoside that is part of the human P blood group antigen (8). P fimbriae determinants from different strains show a high degree of genetic relatedness (18, 26, 43).

Another type of fimbria, termed S fimbriae or S fimbrial adhesins (Sfa), binds to sialic acid-containing receptors (14, 21, 37). These fimbriae are significantly associated with *E. coli* strains that cause septicemia and meningitis. Sfa are especially produced by strains exhibiting the K1 antigen, such as O18:K1 or O83:K1 isolates (15, 34). By using experimental animal model systems, it was demonstrated that S fimbriae contribute to the pathogenicity of the bacteria (4, 29). *Sfa* from different *E. coli* strains are serologically related. Furthermore, their corresponding genetic determinants show strong homologies to each other (34). Previously, it could be shown that S fimbriae and F1C fimbriae (42), which are devoid of any detectable receptor specificity, are homologous with respect to their genetic determinants (36), and both types of fimbria seem to be members of the same distinct group of fimbrial antigens (35).

In this study, we describe the cloning and characterization of a new type of fimbria expressed by an *E. coli* blood culture isolate of serotype O75:K1:H7. These fimbriae show serological cross-reactivity with Sfa-specific antiserum but do not exhibit any hemagglutination. In addition, their corresponding genetic determinant shows a high degree of sequence homology to the Sfa- and F1C-coding gene clusters. Therefore this type of fimbria is termed S/F1C-related fimbriae (Sfr).

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* BK658 (O75:K1:H7) is a blood culture isolate and was obtained from the Institut für Medizinische Mikrobiologie und Immunologie, Ruhr-Universität Bochum, Bochum, Federal Republic of Germany. *E. coli* K-12 strain HB101 was used as the recipient for transformation. In the minicell system, strain DS410 was used. The bacteria were grown in the presence of 50 μg of ampicillin per ml. The recombinant plasmids used are listed in Table 1.

Construction of recombinant plasmids. Chromosomal DNA of *E. coli* BK658 was purified as previously described (12), partially digested with *Sau*3A, and separated on a 0.7% agarose gel. Fragments of 10 to 23 kilobase pairs (kb) were

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TABLE 1. Characterization of strains and clones

Strain or clone	Agglutination with[a]: Guinea pig RBCs	Bovine RBCs	Anti-Sfa antiserum	Fimbria(e) present on cells
BK658 (075:K1:H7)	+++	−	++	F1A, F1B, Sfr
HB101(pMMP658-6)	−	−	++	Sfr
HB101(pANN801-13)	−	+++	+++	Sfa
HB101(pPIL110-54)	−	−	++	F1C

[a] +++, Strong agglutination; ++, agglutination; −, no agglutination. RBCs, erythrocytes.

isolated from the gel by electroelution and ligated into the *Bam*HI site of pBR322 as described by Maniatis et al. (19). Recombinant plasmids were transformed in *E. coli* HB101 by the $CaCl_2$ procedure (16). The presence of insert DNA was checked by isolation and suitable digestions of the recombinant plasmid DNAs, followed by agarose gel electrophoresis (1). Restriction enzymes, pBR322 DNA, and T4 ligase were obtained from Boehringer GmbH, Mannheim, Federal Republic of Germany.

Screening of transformants. Transformants were screened for the presence of fimbriae with antiserum raised against purified fimbriae of strain BK658. Screening was done by the colony blot method as described by Karch et al. (11), except that the bacteria were not treated with polymyxin.

Preparation of antisera. New Zealand White rabbits were immunized subcutaneously with 0.5 mg of purified fimbriae in complete Freund adjuvant. After 4 weeks, immunization was repeated, and after another 2 weeks, sera were collected and stored in aliquots at −20°C.

Purification of fimbriae. Fimbriae were isolated by the method of Karch et al. (9).

Gel electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done as described by Lugtenberg et al. (17), except that the running gel contained 11% polyacrylamide. The samples were boiled for 5 min in 2% SDS–1% β-mercaptoethanol at pH 6.8 prior to application to the gel.

Electron microscopy. Bacteria that were grown overnight in Mueller-Hinton broth were collected by centrifugation (2,000 × *g*, 4°C, 10 min) and suspended in phosphate-buffered saline (PBS) to a concentration of 10^9 bacteria per ml. One drop of this suspension was layered on a 1% Formvar-coated grid. After 5 min, the drop was removed by the aid of a filter paper and the remaining bacteria were negatively stained with 1% uranyl acetate for 1.5 min. The grids were washed twice with distilled water for 30 s and examined in a Zeiss transmission electron microscope.

Hemagglutination. The strains were grown on solid or in liquid media, suspended in PBS (approximately 10^{10} bacteria per ml), and mixed with an equal volume of erythrocytes (5 × 10^8 erythrocytes per ml in PBS). Hemagglutination was tested with human, bovine, guinea pig, sheep, horse, chicken, dog, and African green monkey erythrocytes (the last obtained from Flow Laboratories, Meckenheim, Federal Republic of Germany).

Preparation of minicells and analysis of plasmid-encoded proteins. Plasmid-encoded proteins were expressed in minicells of *E. coli* DS410. Preparation of minicells was performed by sucrose density centrifugation as previously described (41). Purified minicells were labeled with 10 μCi of a ^{14}C-amino acid mixture (Amersham, Braunschweig, Federal Republic of Germany). After being labeled for 2 h at 37°C, minicells were collected in a Eppendorf centrifuge, suspended in 100 μl of distilled water, and stored in aliquots at −20°C. For SDS-PAGE, 10-μl aliquots were incubated with the same volume of 2× sample buffer for 3 min at 100°C. Proteins were separated by SDS-PAGE. The gel was fixed in 7% acetic acid, bathed for a further 20 min in Amplify (Amersham), and subsequently dried. For detection of radioactive proteins, the dried gel was layered on an X-ray film (Fudji RX safety) and incubated for 48 to 92 h at −80°C.

Western blot (immunoblot) analysis. The reaction of fimbriae with anti-fimbrial antisera in immunoblot was done as described by Karch et al. (10).

Quantitative enzyme-linked immunosorbent assay. Purified fimbriae (3 μg per ml of 0.2 M sodium carbonate buffer [pH 9.6]) were coated for 2 h at 37°C on polystyrene microdilution plates. Nonspecific protein binding sites were saturated with 1% bovine serum albumin in PBS for 1.5 h at 37°C, and the fimbriae were incubated with antisera (diluted in PBS plus 1% bovine serum albumin plus 0.05% Tween 20) for 1.5 h at 37°C. The plates were washed thrice with PBS plus 0.05% Tween 20 and incubated for 1 h with alkaline phosphate-conjugated goat anti-rabbit immunoglobulin G (Nordic) that was diluted in PBS-bovine serum albumin-Tween. The plates were washed thrice with PBS-Tween and incubated with substrate buffer containing 0.05% (wt/vol) *o*-phenyldiamine and 0.005% (vol/vol) hydrogen peroxide in phosphate-citrate buffer (pH 5.0). After 30 min at room temperature, the reaction was quantitated by determining the optical density at 450 nm.

Nick translation, hybridization, and autoradiography. As a DNA probe, the 9-kb *Eco*RV fragment of the plasmid pANN801-13, which spans over the whole *sfa* determinant of strain 536 (5), was used. The *Eco*RV fragment was eluted from agarose gels, labeled by nick translation with a mixture of all four α-^{32}P-labeled deoxynucleoside triphosphates as described by Rigby et al. (38), and purified by ethanol precipitation. After cleavage of DNAs with restriction enzymes and separation of the fragments by agarose gel electrophoresis (0.7% agarose), the DNA fragments were transferred to nitrocellulose filters (40). Washing and autoradiography of the filters after hybridization were performed as described previously (34). The filters were hybridized in 50% formamide at 43°C for 3 days. Stringent conditions were used for the washing procedure. Radiochemicals were purchased from New England Nuclear Corp., Boston, Mass.

RESULTS

Fimbriae of strain BK658. The blood culture isolate *E. coli* BK658 (O75:K1:H7) agglutinates guinea pig erythrocytes and yeast cells (13) in a mannose-sensitive manner, indicating the presence of type 1 fimbriae (serotype F1A). In addition, the strain carries F1B fimbriae (F. Ørskov and I. Ørskov, personal communication) and a third type of fimbria which consists of protein subunits of 17 kilodaltons (kDa) in size and cross-reacts with a monospecific antiserum raised against a preparation of Sfa (see Table 1). To characterize this special fimbrial type more precisely and to investigate the genetic relationship between these fimbriae, Sfa and F1C fimbriae, the respective genetic determinant was cloned in *E. coli* HB101. As is shown in this paper, these fimbriae are immunologically as well as genetically related to S and F1C fimbriae, and therefore they are preliminarily termed S/F1C-related fimbriae (Sfr).

Cloning and characterization of the genetic determinant coding for Sfr. Chromosomal *Sau*3A DNA fragments (10 to 23 kb) of strain BK658 were ligated in the *Bam*HI site of the plasmid vector pBR322. The recombinant plasmids were transformed into the *E. coli* K-12 strain HB101. HB101 transformants which were ampicillin resistant were screened for the expression of fimbriae by using antiserum raised against purified fimbriae of the wild-type strain BK658. One transformant, HB101(pMMP658-6), that strongly reacted with the antiserum was isolated and further examined. It could be shown by electron microscopy (Fig. 1) that this clone expresses fimbriae of 1.2-μm lengths and diameters of approximately 7 nm. SDS-PAGE analysis of these fimbriae revealed a subunit molecular weight of 17,000 (Fig. 2). These fimbrial subunits were also detected in fimbrial preparations of strain BK658. The cloned fimbriae failed to react with antiserum against F1A and F1B fimbriae (Ørskov and Ørskov, personal communication) but reacted in an immunoblot with anti-S fimbrial antiserum (for details, see Fig. 5). Furthermore, neither the transformant nor purified cloned fimbriae were able to cause any hemagglutination with erythrocytes of different species, including human and bovine erythrocytes, which possess common specific receptors for Sfa (14, 34).

Physical structure of the *sfr* genetic determinant and its comparison with the gene clusters coding Sfa and F1C fimbriae. The recombinant plasmid pMMP658-6 has a size of 17.2 kb. It consists of the vector pBR322 and an insert DNA of 12.9 kb. A physical map of the insert DNA on the basis of the enzymes *Eco*RI, *Eco*RV, *Cla*I, *Pst*I, and *Sph*I is given in Fig. 3. The map of the *sfr* determinant is compared with the restriction maps of the gene clusters coding for Sfa (*sfa*) and F1C fimbriae (*foc*). Structural and functional similarities between the *sfa* and *foc* determinant were recently described (35), and both determinants exhibit common restriction sites with the *sfr* cluster, suggesting a structural relatedness.

To describe the similarity between the *sfr*, *sfa*, and *foc* determinants more precisely, the plasmids pMMP658-6, pANN801-13, and pPIL110-54, coding for Sfr, Sfa, and F1C, respectively, as well as chromosomal DNA of strain BK658 (to demonstrate simultaneously that the cloned insert DNA of pMMP658-6 is also present in the wild-type DNA), were cleaved with the restriction enzyme *Pst*I and hybridized with an α-^{32}P-labeled *Eco*RV *sfa* gene probe of strain 536 (Fig. 4). It was shown previously (5) that the *sfa* determinant is cleaved by *Pst*I into six fragments designated P5 (2.6 kb), P9 (1.3 kb), P8 (1.35 kb), P11 (0.7 kb), P12 (0.5 kb), and P4 (2.9 kb). The *foc* determinant consists of seven *Pst*I fragments (36, 41) whereas the *sfr* determinant is cleaved into five fragments. It is obvious from Fig. 4 that the *sfa* gene probe strongly hybridized not only with homologous DNA fragments of plasmid pANN801-13 but also with DNA fragments of plasmid pMMP658-6, of the chromosome of BK658, and of the plasmid pPIL110-54, indicating strong sequence homologies of the *sfa* gene probe with these DNAs. The DNAs of pMMP658-6 and BK658 hybridized in fragments of comparable sizes, indicating the presence and identity of the *sfr* determinant in both DNAs. Differences between pMMP658-6 and BK658 DNA were detected for the P4* fragment which, in the case of pMMP658-6, carries vector-specific DNA. An additional fragment in the BK658 DNA is visible which seems to mark a cross-hybridization (presumably via DNA of the control region; see reference 34) with another fimbrial determinant in the BK658 genome.

It is obvious, furthermore, that the fragments P8, P9, and P11 are of identical sizes in the *sfr*, *sfa*, and *foc* determinants. In addition, the P5 fragments of the *sfr* and *sfa* determinants (P5* and P5, respectively) are of nearly identical sizes. In contrast to *sfa* and *foc* gene clusters, *sfr*-specific DNA lacks fragment P12. The P12-specific DNA of *sfr* seems to be fused to P4, resulting in a larger fragment termed P4*.

Serological relationship between Sfr, Sfa, and F1C. To analyze the serological relationship between Sfr, Sfa, and F1C fimbriae, Western blots were performed. As demonstrated in Fig. 5, all three fimbriae reacted with anti-Sfa antiserum. In contrast, anti-Sfr- and anti-F1C-specific antisera reacted only with their corresponding antigens. These results could be obtained not only with denaturated fimbrial subunits but also with native fimbriae in a quantitative enzyme-linked immunosorbent assay. As shown in Table 2, anti-Sfa antiserum gave a strong reaction with Sfa and a weaker reaction with Sfr and F1C fimbriae. In contrast, anti-Sfr and anti-F1C antisera gave a specific reaction with their corresponding antigens only. It can be concluded therefore that all three fimbriae, S, Sfr, and F1C, exhibit common epitopes (expressed in immunogenic form by S fimbriae only) as well as type-specific antigenic determinants.

Characterization of *sfr*-specific gene products in minicells. For most fimbriae, it could be shown that the corresponding genetic determinant codes for the fimbrial subunit and for a set of proteins involved in transport and assembly of the fimbriae on the outer membrane of the bacterium (23, 25). To characterize the proteins encoded by the S-related fimbrial determinant, the recombinant plasmid pMMP658-6 was expressed in minicells. Labeling of the plasmid-encoded proteins with a ^{14}C-amino acid mixture led to the detection of six proteins with sizes of 87, 31, 23, 17, 14.3, and 13.8 kDa, as well as proteins expressed by the vector (Fig. 6). The 17-kDa band most probably represents the fimbrial subunit, as the cloned fimbriae are composed of polypeptides with this molecular size.

DISCUSSION

In this paper, we report the cloning and characterization of a new type of fimbria produced by the *E. coli* blood culture isolate BK658 of serotype O75:K1:H7. These fimbriae show serological cross-reactivity with S fimbriae and F1C fimbriae but fail to exhibit S-specific binding properties as indicated by the negative outcome of hemagglutination tests. Because of striking similarities between the gene clusters coding for the cloned fimbriae and S fimbriae, as well as F1C fimbriae, the new fimbriae were termed S/F1C-related fimbriae (Sfr).

Another fimbrial adhesin found to be associated with O75:K5 *E. coli* strains is the Dr hemagglutinin (previously termed the O75X fimbria-like adhesin) which was cloned recently (27, 28). It is obvious from the different genetic and morphological characteristics of both fimbriae that the Dr hemagglutinin determinant and the *sfr* gene cluster code for two nonrelated types of cell wall appendices present on *E. coli* O75 strains.

TABLE 2. Immunological cross-reactions between cloned fimbriae in an enzyme-linked immunosorbent assay

Antiserum	Titer[a] of antiserum against solid-phase fimbrial antigen		
	Sfr	Sfa	F1C
Sfr	900	7	7
Sfa	40	900	25
F1C	2	9	380

[a] The titer of the antisera are given as the reciprocal ($\times 10^{-2}$) of the highest dilution of the sera giving an A_{450} of 1.

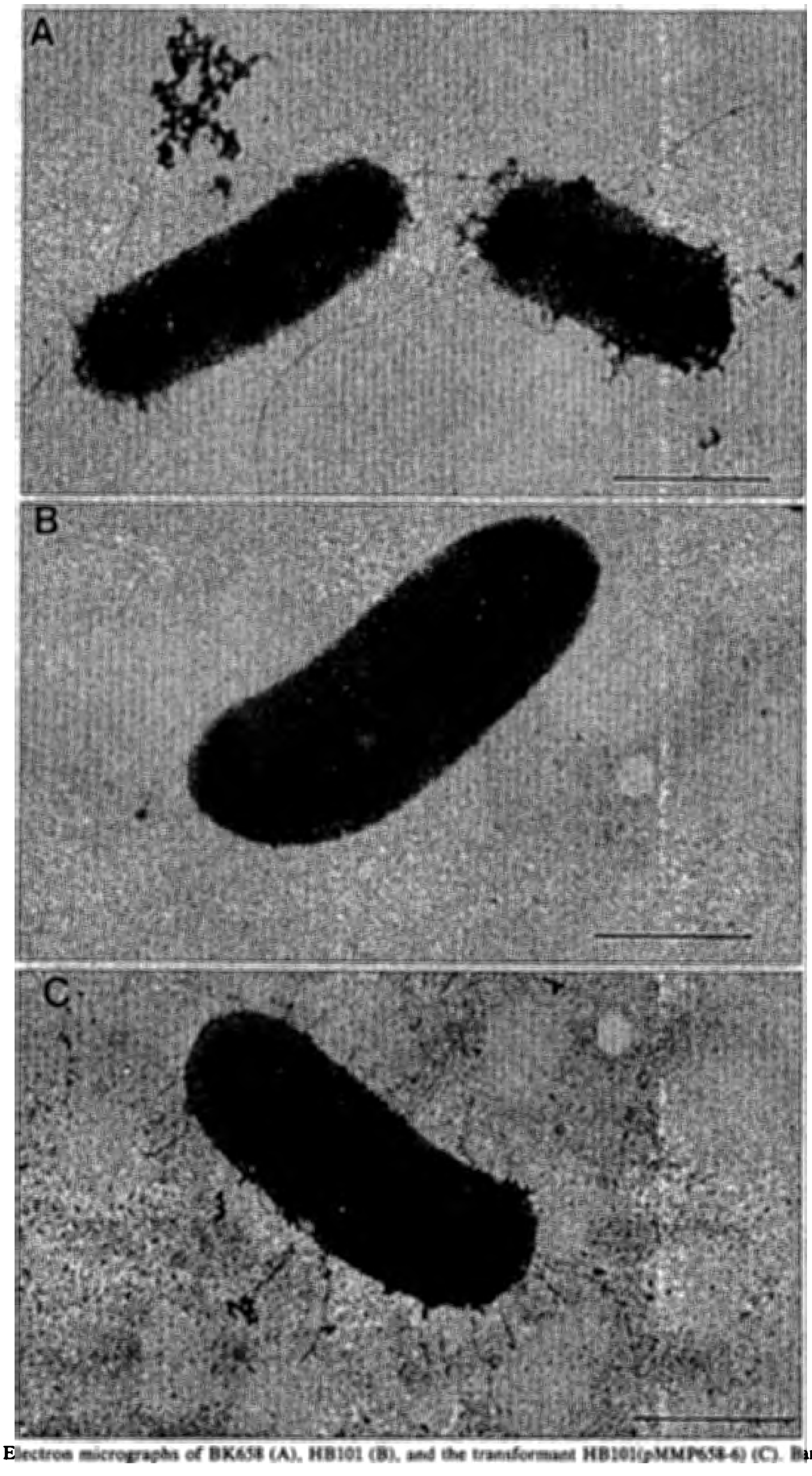

FIG. 1. Electron micrographs of BK658 (A), HB101 (B), and the transformant HB101(pMMP658-6) (C). Bar, 1 μm.

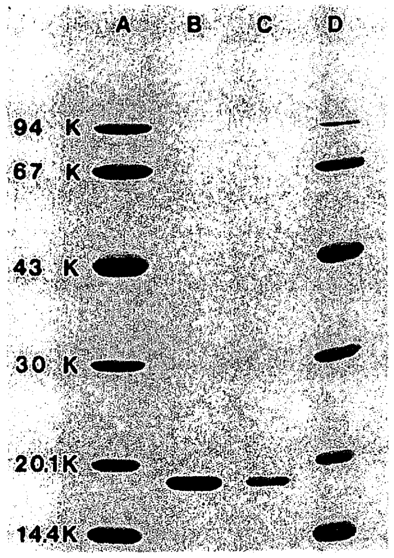

FIG. 2. SDS-PAGE of fimbriae of strains BK658 (lane B) and HB101(pMMP658-6) (lane C). Lanes A and D represent marker proteins. K, Kilodaltons.

In the last few years, several other fimbrial determinants have been cloned from the chromosome of extraintestinal *E. coli* strains (for a review, see reference 26). Some of these determinants, as well as their fimbrial adhesins, show strong similarities with respect to their genetic characteristics and to their binding specificities. Such a relatedness was demonstrated for different P fimbrial determinants which all code for Gal-Gal-binding adhesins (18, 43). In addition, gene clusters coding for type 1 (MS) fimbriae of different strains exhibit similar properties (2). Furthermore, it was shown that Sfa and F1C fimbriae are very similar in several aspects but that they lack a common receptor specificity (35, 36).

The *sfr* determinant is also similar to large regions of the *sfa* and *foc* gene clusters. Otherwise, the binding properties are different from each other. Whereas Sfa agglutinate bovine erythrocytes, no hemagglutinating activity could be detected for Sfr. The reason for the inability of Sfr to agglutinate erythrocytes has not yet been determined. The fact that large deletions were not detectable in the *sfr* determinant after comparison with *sfa* and *foc* argues for the occurrence of point mutations in the adhesin-coding gene or for the presence of binding specificities which are not detectable by agglutination of erythrocytes. The latter was

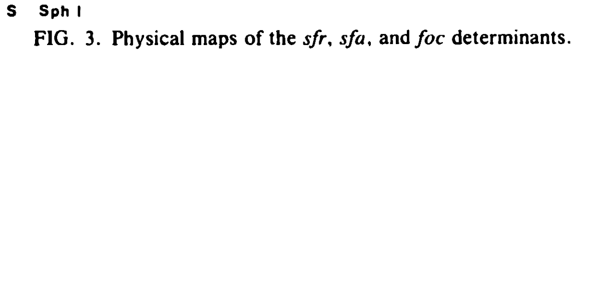

FIG. 3. Physical maps of the *sfr*, *sfa*, and *foc* determinants.

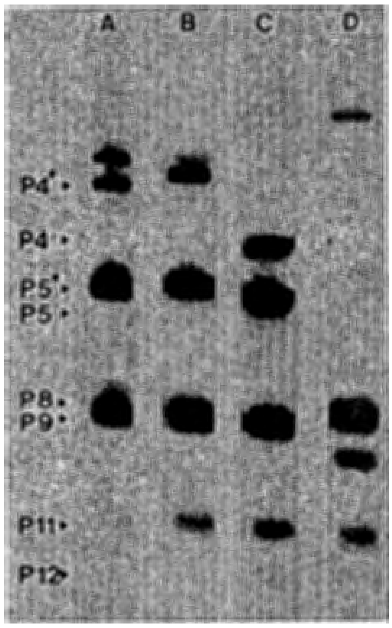

FIG. 4. Southern hybridization patterns of *Pst*I-cleaved DNA of *E. coli* BK658 (lane A) and plasmid DNAs pMMP658-6 (lane B), pANN801-13 (lane C), and pPIL110-54 (lane D). The DNAs were hybridized with an α-^{32}P-labeled *Eco*RV fragment of the *sfa* determinant. The different *Pst*I fragments are indicated as P4 to P12 (see text).

also found for F1C fimbriae, which also lack hemagglutinating activity but seem to be able to bind to human kidney and bladder cells (T. K. Korhonen, personal communication). A similar situation was reported for the 987P pili which are produced by enterotoxigenic *E. coli* strains (24) and which do not agglutinate erythrocytes but seem to bind to a well-characterized receptor present on intestinal brush border cells (3). While differences between Sfa, Sfr, and F1C fimbriae exist in their binding specificities, their corresponding genetic determinants are rather homologous, as demonstrated by the occurrence of similar restriction maps. The genetic relatedness of the three determinants, *sfr*, *sfa*, and *foc*, is further confirmed by the fact that homologous DNA sequences were found along the entire coding regions by Southern hybridization. This is in contrast to P fimbrial determinants and *sfa* gene clusters, which share homology only in the control regions of the determinants (36). However, minor differences between *sfr*, *sfa*, and *foc* exist in the regions which code for the fimbrial subunit in the *sfa* determinant. The corresponding *sfa*-specific *Pst*I fragment P12 is not present in *sfr* DNA preparations, indicating sequence alterations in the fimbrillin proteins. These alterations might explain the different antigenic properties of the fimbrial types. In addition, differences seem to exist in the flanking sequences of the determinants which are marked by the different sizes of the *Pst*I fragments specific for these regions (P4, P5: Fig. 4).

The *sfr* determinant, which is located on a 12.9-kb DNA fragment in the pBR322 derivative pMMP658-6, codes for a minimum of six different proteins (Fig. 6). One of these proteins, 17 kDa in size, represents the fimbrillin subunit molecule. The function of the other proteins remains to be determined, but it can be speculated that they are involved in transport and biogenesis processes of the Sfr, as already

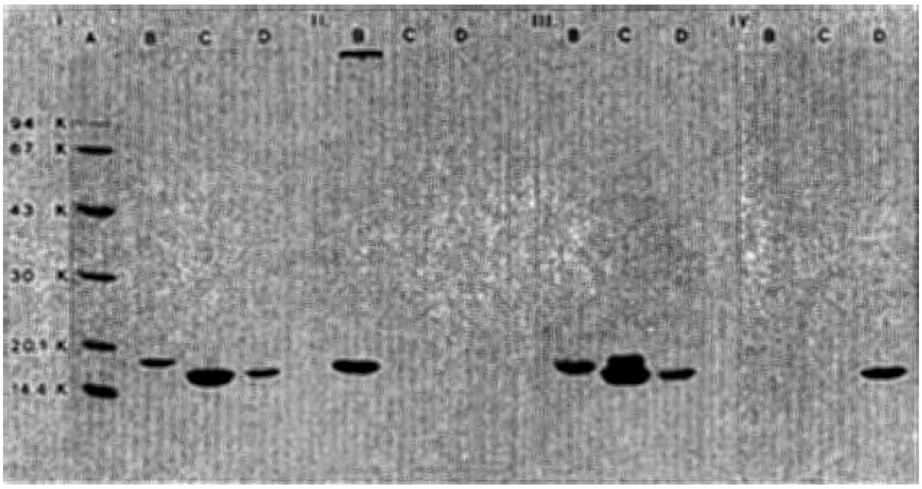

FIG. 5. SDS-PAGE (I) and Western blots (II through IV) of fimbriae. Lanes B, Sfr; lanes C, Sfa; lanes D, F1C; lanes A, marker proteins. The fimbriae were probed against anti-Sfr antiserum (II), anti-Sfa antiserum (III), and anti-F1C antiserum (IV). K, Kilodaltons.

demonstrated for several other fimbrial adhesins, including P and type 1 fimbriae (23, 25, 26, 31). The size of the Sfr protein subunit molecules (17 kDa) resembles those of the fimbrillin proteins of S fimbriae and F1C fimbriae (16 to 17 kDa; see references 21 and 39). The similarities of the subunit proteins of Sfr, Sfa, and F1C fimbriae were also demonstrated by serological cross-reactions with anti-Sfa antiserum. These data indicate the occurrence of common epitopes in all three antigens. Similar results for S fimbriae and F1C fimbriae with respect to their serologic relatedness were obtained recently by Ott et al. (35) with monoclonal anti-Sfa- and anti-F1C-specific antibodies.

Our data presented here confirm and extend the observation that Sfa and F1C fimbriae belong to one particular group of fimbrial antigens. Additionally, we present evidence of a third, S/F1C-related fimbrial type which belongs to the same group of fimbrial determinants as the gene clusters coding for Sfa and F1C fimbriae.

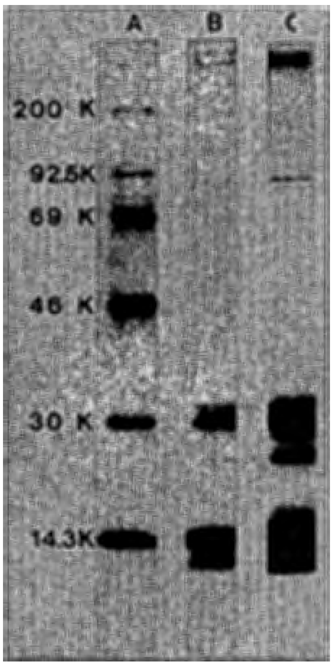

FIG. 6. Autoradiogram of ^{14}C-labeled proteins encoded by the plasmid pMMP658-6. Lane A, Marker proteins; lane B, ^{14}C-labeled proteins after 48 h of exposure of the SDS gel to an X-ray film; lane C, proteins after 72 h of exposure of the gel to the film. K, Kilodaltons.

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